AMENDMENTS TO THE SPECIFICATION

Please replace paragraphs 190, 214, 248, 278, 284, 333, 341 350, 351, 353, 354, 359, 363, 381, 387, 493, and 494 with the following amended paragraphs. Applicants note that the amendments, which are formal in nature, do not introduce new matter.

[0190] The invention provides a variety of caged sensors and regulators. For example, a first group of caged sensors, regulators and compounds is designed for use with protein kinases. Protein kinase substrates are ideal in cell sensor probes, since all signal transduction is regulated by this superfamily of enzymes (and ATP is the origin of all energy exchanged). Protein kinases represent the largest super family of homologous proteins with over 500+ (and possibly as many as 1000 or more) different mammalian members known to date. An even greater number is predicted from genome sequencing and analysis. *See*, e.g., Manning et al. "The Protein Kinase Complement of the Human Genome" Science 298: 1912-1934 (2002); the internet at ~~www.~~cellsignal.com/reference/pathway; and~~,~~ the internet at ~~www.~~cellsignal.com/reference/kinase/index.asp.

[0214] In one embodiment, phosphorylation of the substrate results in binding of a phosphobinder to the phosphorylated substrate, the binding of the phosphobinder preventing the interaction of the first and the second label or the quencher. Optionally, the phosphobinder is associated with one or more second caging groups, the presence of which prevents the phosphobinder from binding the phosphorylated substrate. Indeed, one aspect of the invention is simply a caged phosphobinder. The second caging groups are optionally removable under different conditions than the first caging groups inhibiting phosphorylation of the substrate. In one embodiment, the phosphobinder is an antibody. Such antibodies are well known in the art, and many are commercially available, e.g., from Zymed Laboratories, Inc. or QIAGEN (www.qiagen.com). In other embodiments, the phosphobinder comprises an SH-2 domain, a PTB domain, a 14-3-3 domain, an FHA domain, a WD40 domain and/or a WW domain. These and other such domains are reviewed in e.g., Yaffe and Elia "Phosphoserine/ threonine-binding domains" Curr Opin Cell Biol. (2001) 13(2):131-8; Li et

al. "The FHA domain mediates phosphoprotein interactions" J Cell Sci. (2000) 113 Pt 23:4143-9; the internet at ~~www.~~cellsignal.com/reference/domain/index.asp; and the internet at ~~www.~~mshri.on.ca/pawson/ww.html.

[0248] Optionally, the caged MB can comprise one or more nonnatural nucleotides. For example, the nonnatural nucleotides can be included to increase nuclease resistance and/or to enhance specificity of the beacon. As one example, the stem of the MB can comprise, at complementary positions of the two strands of the stem, at least one pair of nonnatural nucleotides that base pair with each other but that do not Watson-Crick base pair with the bases typical to biological DNA or RNA (i.e., A, C, G, T, or U). Uncaged molecular beacons comprising such nonnatural nucleotides in their stem are also a feature of the invention. Examples of nonnatural nucleotides include, but are not limited to, Locked NucleicAcid™ nucleotides (available from Exiqon A/S, on the internet at ~~www.~~exiqon.com; *see*, e.g., SantaLucia Jr. (1998) Proc Natl Acad Sci 95:1460-1465) and isoG, isoC, isoA, isoT, and other nucleotides used in the AEGIS system (Artificially Expanded Genetic Information System, available from EraGen Biosciences, on the internet at ~~www.~~eragen.com; *see*, e.g., USPN 6,001,983, USPN 6,037,120, and USPN 6,140,496).

[0278] Caging the interfering RNA allows, e.g., precise control over the timing of gene silencing by controlling initiation of RNA interference (also called RNAi or RNA-mediated interference). Use of RNAi for inhibiting gene expression in a number of cell types (including, e.g., mammalian cells) and organisms is well described in the literature, as are methods for determining appropriate interfering RNA(s) to target a desired gene and for generating such interfering RNAs. For example, RNA interference is described e.g., in US patent application publications 20020173478, 20020162126, and 20020182223 and in Hannon G.J. "RNA interference" Nature. 2002 Jul 11, 418 (6894):244-51; Ueda R. "RNAi: a new technology in the post-genomic sequencing era" J Neurogenet. 2001, 15(3-4):193-204; Ullu et al. "RNA interference: advances and questions" Philos Trans R Soc Lond B Biol Sci. 2002 Jan 29, 357(1417):65-70; and Schmid et al. "Combinatorial RNAi: a method for evaluating the functions of gene families in Drosophila" Trends Neurosci. 2002 Feb, 25

(2):71-4. A kit for producing interfering RNAs is commercially available, e.g., from Ambion, Inc. (on the internet at ~~www.~~ambion.com, the Silencer™ siRNA construction kit). Kits for labeling such RNAs are available from the same source.

[0284] Caged compounds, including the caged sensors and regulators of this invention, can be used in various combinations. Thus, in one class of embodiments, the invention provides a composition comprising at least a first caged component and a second caged component. Typically, the first caged component can be uncaged by exposure to energy of a first type and the second caged component can be uncaged by exposure to energy of a second type different from the first type. The various uncaging energies for the first and second types can be the same general type (e.g., where the energy of the first type is light of a first wavelength and the energy of the second type is light of a second wavelength) or they can be of different general types (e.g., light and heat or light and sonication energy). Alternatively, the first caged component and the second caged component can be uncaged by exposure to energy of the same type (e.g., the first and second caged components can be uncaged by light of a given wavelength). As noted, caged components can also be uncaged by changes in pH or ionic strength or by other environmental changes. The composition optionally comprises three or more caged components. The caged components can be, e.g., any of those noted above, e.g., a caged sensor (e.g., an enzyme or binding sensor), a caged nucleic acid probe, a caged modulator, a caged antisense nucleic acid, a caged ribozyme, a caged biomolecular analog, a caged transcription factor, a caged molecular decoy, a caged antibody, a caged aptamer, and/or a caged interfering RNA or RNAi-based sensor (e.g., as described above or in U.S. Patent Application 60/484,785, filed July 3, 2003 and in U.S. Patent Application 10/716,393~~/~~_____ (attorney docket number 70-000410US), filed of even date herewith, entitled "RNAi-based sensors, caged interfering RNAs, and methods of use thereof" by Nguyen and McMaster). Other caged components include, but are not limited to, a caged polypeptide, a caged nucleic acid, a caged lipid, a caged carbohydrate, a caged small molecule, a caged metal ion, a caged nucleotide (e.g., a caged nucleoside triphosphate or caged cAMP), a caged chelating agent, a caged fluorescent dye, a caged second messenger, and/or a caged neurotransmitter. *See*, e.g., Haughland (2003) Handbook

of Fluorescent Probes and Research Products Ninth Edition or the current Web Edition, available from Molecular Probes, Inc.; and Shigeri et al. (2001) "Synthesis and application of caged peptides and proteins" Pharmacology & Therapeutics 91:85-92). A number of caged compounds, including, for example, caged nucleotides, caged Ca2+, caged chelating agents, caged neurotransmitters, and caged luciferin, are commercially available, e.g., from Molecular Probes, Inc. (on the internet at (www.) ~~www.~~molecularprobes.com). The caged components can be in essentially any combination; e.g., two or more caged sensors, a caged sensor and a caged modulator, a caged sensor and a caged chelating agent, a caged interfering RNA and a caged nucleic acid probe, two or more caged nucleic acid probes, a caged antisense nucleic acid and a caged nucleic acid probe, a caged sensor and a caged nucleic acid probe, a caged sensor and a caged interfering RNA and a caged nucleic acid probe, or a caged transcription factor and a caged nucleic acid probe, to list only a few examples. The compositions can be formulated in any of the various states noted above, e.g., in cells, cell compartments, cell extracts, matrices, systems, and devices and kits.

[0333] The detection reagent can be any moiety that facilitates detection of the modified substrate. Examples include phosphobinders, antibodies that specifically bind to the modified substrate, nucleic acids that specifically bind to the modified substrate, aptamers that specifically bind to the modified substrate, micro or nano particles that bind to the modified substrate, or a combination thereof. The signal that is produced is detected by the appropriate method, e.g., performing a homogeneous assay, a heterogeneous assay, FRET, Q-FRET, TR-FRET, and/or fluorescence polarization. FRET (Fluorescence Resonance Energy Transfer) is a non-radiative energy transfer phenomenon in which two fluorophores with overlapping emission and excitation spectra, when in sufficiently close proximity, experience energy transfer by a resonance dipole induced dipole interaction. The phenomenon is commonly used to study the binding of analytes such as nucleic acids, proteins and the like. FRET is a distance dependent excited state interaction in which emission of one fluorophore is coupled to the excitation of another which is in proximity (close enough for an observable change in emissions to occur). Some excited fluorophores interact to form excimers, which are excited state dimers that exhibit altered emission spectra

(e.g., phospholipid analogs with pyrene sn-2 acyl chains). *See*, e.g., Haugland (1996) Handbook of Fluorescent Probes and Research Chemicals Published by Molecular Probes, Inc., Eugene, OR. e.g., at chapter 13). For example, in one embodiment, the substrate of interest can comprise a first label and the detection reagent can comprise a second label, wherein, for example, the first label comprises an acceptor FRET moiety and the second label comprises a donor FRET moiety. The acceptor moiety comprises a quencher moiety, wherein the quencher moiety can be selected from, e.g., a group consisting of: fluorophores, Dabsyl, Black-hole™, QSY™, and an Eclipse Dark Quencher. Similarly, the donor moiety is optionally selected from a group consisting of: Xanthene dyes, Cyanine dyes, Metal-Ligand Complexes, Coumarin dyes, BODIPY dyes, and Pyrene dyes, and the like. *See also*, Haugland (2003) Handbook of Fluorescent Probes and Research Chemicals Ninth Edition Published by Molecular Probes, Inc., Eugene, OR. In Q-FRET, when a quencher such as Dabcyl and a fluorophore such as coumarin are located near each other, their proximity guides the emission of the fluorophore. See, e.g., Dubertret et al. (2001) Nature Biotech. 19:365-370; see also the internet at ~~www.~~prozyme.com and ~~www.~~IDTdna.com.

[0341] It will be appreciated that a related or additional system of the invention includes a cell comprising a caged substrate of interest and a modification component, an actual or potential modulator of the modification component, a source of uncaging energy (light, heat, pH, etc.), a cell lysis or fixation module, a reporter that detects modifications to the substrate of interest, and a detector that detects a signal from the reporter. The various system components can be any of those noted above in the context of the methods. The lysis or fixation module can include high throughput fluid handling components such as conventional robotic and/or pipettor systems, and/or microfluidic systems for transferring cells, lysis or fixation reagents, or the like. Many automated high throughput systems are commercially available and can be adapted to the present invention by including the appropriate system elements as set forth herein. For example, a variety of automated systems are available from the Zymark Corporation (Zymark Center, Hopkinton, MA), which utilize various Zymate systems (*see also* the internet at ~~www.~~zymark.com), which typically include, e.g., robotics and fluid handling modules. Similarly, the common ORCA® robot, which is

used in a variety of laboratory systems, e.g., for microtiter tray manipulation, is also commercially available, e.g., from Beckman Coulter, Inc. (Fullerton, CA). Microfluidic systems have established the potential for automation and laboratory productivity increases as well. In these microfluidic systems, automated fluid handling and other sample manipulations are controlled at the microscale level. Such systems are now commercially available. For example, the Hewlett-Packard (Agilent Technologies) HP2100 bioanalyzer utilizes LabChip™ technology to manipulate extremely small sample volumes. In this "lab-on-a-chip," system, sample preparation, fluid handling and biochemical analysis steps are carried out within the confines of a microchip. The chips have microchannels fabricated, e.g., in glass, providing interconnected networks of fluid reservoirs and pathways. The Caliper High Throughput Screening System (*see*, e.g., the internet at ~~www.~~calipertech.com/products/index.htm) provides an interface between standard library formats and chip technologies (*see, generally,* the internet at ~~http://www.~~calipertech.com).

[0350] In step **6210,** cell **6205** is lysed or fixed to stop the reaction of kinase **6208** on substrate **6207**. Phosphorylation sensitive reporter **6211** is contacted to substrate **6209** to detect the phosphorylation state of substrate **6209**. Various approaches for this detection are shown in **Figure 63**. As shown, homogeneous solution phase assays such as FRET, Q-FRET or TR-FRET (**Panel A**) or fluorescence polarization (**Panel B**), or a heterogeneous assay that comprises a solid phase upon which substrate **6209** is bound, e.g., through a streptavidin-biotin linkage as shown in **Panel C,** can be used. In **Panel A**, reporter **6211** comprises a quencher, Eu or acceptor fluorescent moiety ("F2") while **6209** comprises a donor fluorescent moiety ("F1"). It will be appreciated that F1 and F2 can be reversed in alternate embodiments. Reporter **6211** also comprises phosphobinder domain **6212** (e.g., an antibody, metal ion, affinity chromatography moiety such as IMAC, etc.). In **Panel B**, reporter **6211** does not comprise a separate label, but still comprises phosphobinder domain **6212** and detection is performed, e.g., by measuring a change in rotation brought about by binding, e.g., using fluorescence polarization. **Panel C** shows a heterogenous assay, in which substrate **6209** is bound to streptavidin-coated plate **6213** through a biotin linkage. Reporter **6211** comprises phosphobinder domain **6212** (e.g., an anti-phospho antibody, e.g., detected

with an alkaline phosphatase conjugated secondary antibody and a chemiluminecescent, colorimetric or fluorescent substrate, e.g., a CSPD™ substrate and Sapphire II™ enhancer available from Applied Biosystems, on the internet at ~~www.~~appliedbiosystems.com).

[0351] In certain embodiments, immunofluorescent detection is used, in which cells are fixed and substrate **6209** is detected using a phosphoantibody. Other detection possibilities include polyarginine binding to substrate **6209**, use of unnatural ATP analogs followed by chemical conjugation to biotin (e.g., use of a modified ATP with a thio-R group on the phosphate in the assay results in the kinase transferring a thio-phosphate onto the substrate, such that various reporters such as biotin, fluorescent labels, or enzymes can then be conjugated to the thio group; see, e.g., the internet at ~~http://www.~~chromagen.com/polaris.htm), or various phosphorylation-dependent substrate protection strategies. For example, certain peptide substrates can be protected from protease digestion by incorporation of a phosphate, which will result in maintaining F1 on substrate **6209** (and cleavage of F1 from **6209** in unphosphorylated, unprotected substrates). Similarly, phosphorylated peptides have a difference in charge which permits electrophoretic resolution of substrate **6209** from substrate **6207**. A variety of other methods of detecting phosphorylated substrates are commonly known in the art and can be adapted to practice of the present invention.

[0353] A large number of caging groups, and a number of reactive compounds that can be used to covalently attach caging groups to other molecules, are well known in the art. Examples of photolabile caging groups include, but are not limited to: nitroindolines; N-acyl-7-nitroindolines; phenacyls; hydroxyphenacyl; brominated 7-hydroxycoumarin-4-ylmethyls (e.g., Bhc); benzoin esters; dimethoxybenzoin; meta-phenols; 2-nitrobenzyl; 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE); 4,5-dimethoxy-2-nitrobenzyl (DMNB); alpha-carboxy-2-nitrobenzyl (CNB); 1-(2-nitrophenyl)ethyl (NPE); 5-carboxymethoxy-2-nitrobenzyl (CMNB); (5-carboxymethoxy-2-nitrobenzyl)oxy) carbonyl; (4,5-dimethoxy-2-nitrobenzyl)oxy) carbonyl; desoxybenzoinyl; and the like. *See*, e.g., USPN 5,635,608 to Haugland and Gee (June 3, 1997) entitled "α-carboxy caged compounds"; Neuro 19, 465

(1997); J Physiol 508.3, 801 (1998); Proc Natl Acad Sci USA 1988 Sep, 85(17):6571-5; J Biol Chem 1997 Feb 14, 272(7):4172-8; Neuron 20, 619-624, 1998; Nature Genetics, vol. 28:2001:317-325; Nature, vol. 392,1998:936-941; Pan, P., and Bayley, H. "Caged cysteine and thiophosphoryl peptides" FEBS Letters 405:81-85 (1997); Pettit et al. (1997) "Chemical two-photon uncaging: a novel approach to mapping glutamate receptors" Neuron 19:465-471; Furuta et al. (1999) "Brominated 7-hydroxycoumarin-4-ylmethyls: novel photolabile protecting groups with biologically useful cross-sections for two photon photolysis" Proc. Natl. Acad. Sci. 96(4):1193-1200; Zou et al. "Catalytic subunit of protein kinase A caged at the activating phosphothreonine" J. Amer. Chem. Soc. (2002) 124:8220-8229; Zou et al. "Caged Thiophosphotyrosine Peptides" Angew. Chem. Int. Ed. (2001) 40:3049-3051; Conrad II et al. "p-Hydroxyphenacyl Phototriggers: The reactive Excited State of Phosphate Photorelease" J. Am. Chem. Soc. (2000) 122:9346-9347; Conrad II et al. "New Phototriggers 10: Extending the π,π^* Absorption to Release Peptides in Biological Media" Org. Lett. (2000) 2:1545-1547; Givens et al. "A New Phototriggers 9: p-Hydroxyphenacyl as a C-Terminus Photoremovable Protecting Group for Oligopeptides" J. Am. Chem. Soc. (2000) 122:2687-2697; Bishop et al. "40-Aminomethyl-2,20-bipyridyl-4-carboxylic Acid (Abc) and Related Derivatives: Novel Bipyridine Amino Acids for the Solid-Phase Incorporation of a Metal Coordination Site Within a Peptide Backbone" Tetrahedron (2000) 56:4629-4638; Ching et al. "Polymers As Surface-Based Tethers with Photolytic triggers Enabling Laser-Induced Release/Desorption of Covalently Bound Molecules" Bioconjugate Chemistry (1996) 7:525-8; BioProbes Handbook, 2002 from Molecular Probes, Inc.; and Handbook of Fluorescent Probes and Research Products, Ninth Edition or Web Edition, from Molecular Probes, Inc, as well as the references below. Many compounds, kits, etc. for use in caging various molecules are commercially available, e.g., from Molecular Probes, Inc. (on the internet at ~~www.~~molecularprobes.com).

[0354] A caging group optionally comprises a first binding moiety that can bind to a second binding moiety. For example, a commercially available caged phosphoramidite [1-N-(4,4'-Dimethoxytrityl)-5-(6-biotinamidocaproamidomethyl)-1-(2-nitrophenyl)-ethyl]-2-cyanoethyl-(N,N-diisopropyl)-phosphoramidite (PC Biotin Phosphoramadite, from Glen

Research Corp., on the internet at ~~www.~~glenres.com) comprises a photolabile group and a biotin (the first binding moiety in this example). A second binding moiety, e.g., streptavidin or avidin, can thus be bound to the caging group, increasing its bulkiness and its effectiveness at caging. In certain embodiments, a caged component comprises two or more caging groups each comprising a first binding moiety, and the second binding moiety can bind two or more first binding moieties simultaneously. For example, the caged component can comprise at least two biotinylated caging groups; binding of streptavidin to multiple biotin moieties on multiple caged component molecules links the caged components into a large network. Cleavage of the photolabile group attaching the biotin to the component results in dissociation of the network.

[0359] Caged modulators (e.g., inhibitors and activators), small molecules, etc. can be similarly produced by reaction with caging compounds or by synthesis. *See*, e.g., Trends Plant Sci (1999) 4:330-334; PNAS (1998) 95:1568-1573; USPN 5,888,829 to Gee and Millard (March 30, 1999) entitled "Photolabile caged ionophores and method of using in a membrane separation process"; USPN 6,043,065 to Kao et al. (March 28, 2000) entitled "Photosensitive organic compounds that release 2,5,-di(tert-butyl) hydroquinone upon illumination"; USPN 5,430,175 to Hess et al. (July 4, 1995) entitled "Caged carboxyl compounds and use thereof"; USPN 5872243; and PNAS (1980) 77:7237-41. A number of caged compounds, including for example caged nucleotides, caged Ca2+, caged chelating agents, caged neurotransmitters, and caged luciferin, are commercially available, e.g., from Molecular Probes, Inc. (on the internet at ~~www.~~molecularprobes.com).

[0363] Delivery of molecules by exposing cells to pulses of laser beam (laserfection or laser transfection) has also been described, as have delivery by pinocytosis or use of streptolysin-O (SLO). As another example, a kit from Active Motif utilizing the PEP-1 peptide as a delivery system for proteins ranging from a small peptide to a large IgG antibody is commercially available (Chariot ™, on the internet at ~~www.~~activemotif.com). However, these methods require manipulation of the cells, e.g., adding and removing transfection materials, pre-treating cells, and special apparatus and equipment, etc.

[0381] Other optically detectable labels can also be used in the invention. For example, gold beads can be used as labels and can be detected using a white light source via resonance light scattering. *See*, e.g. the internet at ~~www.~~geniconsciences.com. Suitable non-optically detectable labels are also known in the art. For example, magnetic labels can be used in the invention (e.g., 3 nm superparamagnetic colloidal iron oxide as a label and NMR detection; *see*, e.g., Nature Biotechnology (2002) 20:816-820).

[0387] Oligonucleotides, including modified oligonucleotides (e.g., oligonucleotides comprising fluorophores and quenchers, unnatural nucleotides, 2'-O-methyl nucleotides, and/or phosphorothioate, methylphosphonate, or boranophosphate linkages) can also be ordered from a variety of commercial sources known to persons of skill. There are many commercial providers of oligo synthesis services, and thus, this is a broadly accessible technology. Any nucleic acid can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (on the internet at ~~www.~~mcrc.com), The Great American Gene Company (on the internet at ~~www.~~genco.com), ExpressGen Inc. (on the internet at ~~www.~~expressgen.com), QIAGEN (oligos.qiagen.com), and many others. PNAs can be custom ordered from any of a variety of sources, such as PeptidoGenic (pkim@ccnet.com), HTI Bio-products, Inc. (on the internet at ~~www.~~htibio.com), BMA Biomedicals Ltd (U.K.), Bio·Synthesis, Inc., and many others. A variety of commercial suppliers produce standard and custom molecular beacons, including Cruachem (cruachem.com), Oswel Research Products Ltd. (UK; oswel.com), Research Genetics (a division of Invitrogen, Huntsville AL (resgen.com)), the Midland Certified Reagent Company (Midland, TX; mcrc.com) and Gorilla Genomics, LLC (Alameda, CA).

[0493] The caged PKC sensor was transfected into HeLa cells using Profect P-2 (Targeting Systems, on the internet at ~~www.~~targetingsystems.com), a non-lipid reagent that forms noncovalent complexes with polypeptides, enabling transport of the associated

polypeptides into cells (and optionally nuclei). Profect P-2 also has endosomolytic properties that protect the internalized polypeptides from degradation in the lysosomes.

[0494] To demonstrate cellular delivery and *in vivo* activation of the caged PKC sensor, 10,000 HeLa cells were plated per well in 96 well plates in complete DMEM and incubated at 37°C overnight. The caged PKC sensor was transfected into the cells at a concentration of 10 μM, as follows. To 37.5 μl serum-free DMEM, 2.5 μl Profect P-2 and then 10 μl of caged PKC sensor (diluted to 500 μM in PBS) were added. The reagents were mixed gently and incubated for 30 minutes at room temperature. The DMEM was removed from the well containing the HeLa cells, and the cells were washed once with PBS. After addition of 450 μl serum-free DMEM to the Profect P-2-caged sensor complex, 50 μl of the complex was added to each well. The HeLa cells were incubated with the complex at 37°C for 2 h (or optionally longer, with the addition of fresh DMEM). To uncage the caged PKC sensor, cells were exposed from beneath the plate to 1 J/cm^2 365 nm UV light. Uncaging light was produced by a BlueWave™ UV Spot Light System fitted with a Lightguide mount assembly, Cool Blue™ filter, and Lightguide rod lens assembly (Dymax Corp., on the internet at ~~www.~~dymax.com, part numbers 38600, 38670, and 38699). Wells containing cells in which the sensor was not to be uncaged were masked with aluminum foil. Immediately after uncaging, PMA was added to 1 μM to induce PKC activity. Fluorescent signal from the PKC sensor was read on an Acumen Explorer™ microplate reader.